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Short Peptides in Minimalistic Biocatalyst Design

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Abstract: We review recent developments in the use of short peptides in the design of minimalistic biocatalysts focusing on ester hydrolysis. A number of designed peptide nanostructures are shown to have (modest) catalytic activity. Five features are discussed and illustrated by literature examples, including primary peptide sequence, nanosurfaces/scaffolds, binding pockets, multivalency and the presence of metal ions. Some of these are derived from natural enzymes, but others, such as multivalency of active sites on designed nanofibers, may give rise to new features not found in natural enzymes. Remarkably, it is shown that each of these design features give rise to similar rate enhancements in ester hydrolysis. Overall, there has been significant progress in the development of fundamental understanding of the factors that influence binding and activity in recent years, holding promise for increasingly rational design of peptide based biocatalysts.

Keywords: primary sequence, histidine, nano-surface/scaffold, multivalency, metals, rational design, catalysis, ester hydrolysis.

Introduction

Both chemists and biologists have long been inspired by the efficiency and selectivity of natural enzymes in catalysing wide ranging reactions, some of which are extremely challenging to achieve using non-enzymatic means. This has been the basis for extensive research into elucidating the basic mechanisms of biochemical catalysis as well as the development of biologically inspired synthetic catalysts that may have biotechnological or biomedical uses. The ability of an enzyme to fold into a structure and organise functional groups for catalysis and binding is reliant on its amino acid sequence (and in some cases, the presence of cofactors such as metal ions, porphyrin or flavin adenine dinucleotide, amongst others) [1,2]. The majority of enzymes are greater than one hundred amino acid residues in length. Proteins of this size have a vast number of potential primary sequences capable of folding (thereby producing binding pockets) and function (presenting residues for catalysis) [3,4]. Remarkably, the process of evolution has selected for relatively few stable three-dimensional structures from the vast number of possibilities.

Today's enzymes emerged initially from simpler structures to more developed and complex levels of organisation [5,6]. One common class of hydrolase enzymes contain conserved serine and histidine as active components in their catalytic triad, complemented by an acidic residue such as aspartic or glutamic acid, which directly contribute to peptide bond hydrolysis [7]. The fact that enzymes evolved suggests that simpler functional precursors to hydrolases may exist and these could provide information about the design of short peptide catalysts. Indeed, it has been observed that the dipeptide seryl-histidine (Ser-His) is able to catalyse a number of hydrolytic reactions in water [8], albeit with low selectivity and modest rates.

The use of peptide self-assembly and nanoparticle functionalization approaches opens up new opportunities for catalyst design that are not found in natural enzymes. In this paper we review recent developments in this area and assess the relative importance in enhancing reaction rates of five features that have been explored in biocatalyst design.

Hydrolase Enzyme – the Catalytic Triad

As an example, let's look at a common class of biocatalysts, proteases, which possess the ability to hydrolyse amide (as well as ester) bonds. This amide hydrolysis reaction is extremely challenging with a half-life of 450 years [9,10]. Protease enzymes make use of a catalytic triad (Figure 1a)

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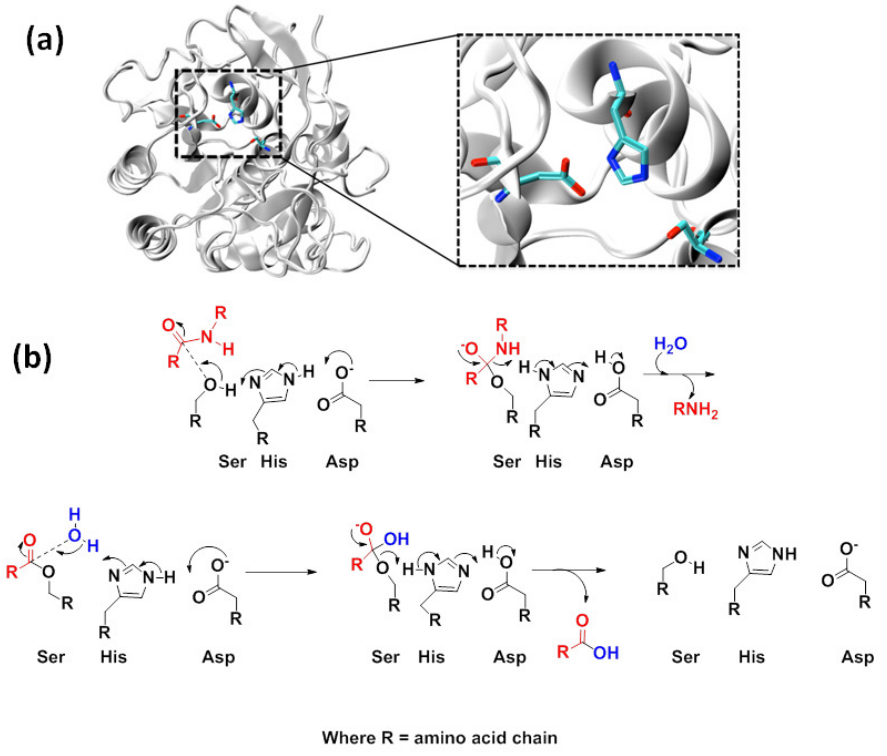


Figure 1. Example of a hydrolase with catalytic triad. (a) Subtilisin with catalytic residues highlighted in molecular detail (PDB 1ST2). (b) Mechanistic outline for the hydrolysis of a peptide bond by a serine protease enzyme.

in the following manner to facilitate this reaction. The serine side chain acts as a nucleophile attacking the carbonyl carbon of an amide or ester substrate whilst the imidazole ring in histidine acts as either a proton donor or acceptor [11]. The acidic residue is used to polarise and align the base which in turn activates the substrate to form the tetrahedral transition state (Figure 1b). The triad residues were identified as crucial components through mutagenesis studies, *i.e.* mutating the serine, histidine or acidic residues resulted in a reduction of activity [7,12]. The most important residue with respect to catalysis is histidine as the catalytic activity dropped most dramatically upon mutation of this residue. In addition to providing the chemical moieties required for chemical catalysis, the three-dimensional structure is fundamental for defining a microenvironment with the right physicochemical properties for catalysis to occur, defining selectivity of the catalyst (through hydrophobic binding pockets), active sites buried inside the protein structure and fixing the conformation of the catalytic site in a dynamic structure, which are all essential for enzymes to function effectively [13].

Other classes of hydrolase enzymes, for example carbonic anhydrase, require the presence of a metal ion (Zn^{2+}) as a cofactor. The mechanism of catalytic activity for these enzymes does not utilise a catalytic triad but coordinates the metal ion in three places by histidine side chains. Water occupies the fourth coordination site causing polarisation of the oxygen-hydrogen bond and weakening it. A fourth histidine residue is positioned close by and accepts a proton leaving a hydroxide attached to the metal. The active site contains a specific pocket for carbon dioxide which brings it close to the hydroxide allowing the hydroxide to attack the carbon dioxide and form bicarbonate [14].

Design Approaches in the Pursuit of Synthetic Catalysts

As discussed, nature evolved impressive catalysts in the form of enzymes; but they may not always be ideally suited for applications or environments that are different from their natural ones. Enzyme (directed) evolution [15-17] has achieved much in improving catalysts but it is not a rational design approach and the proteins produced are still complex. It is intriguing to consider whether simpler yet efficient synthetic, low-molecular weight catalysts can be produced.

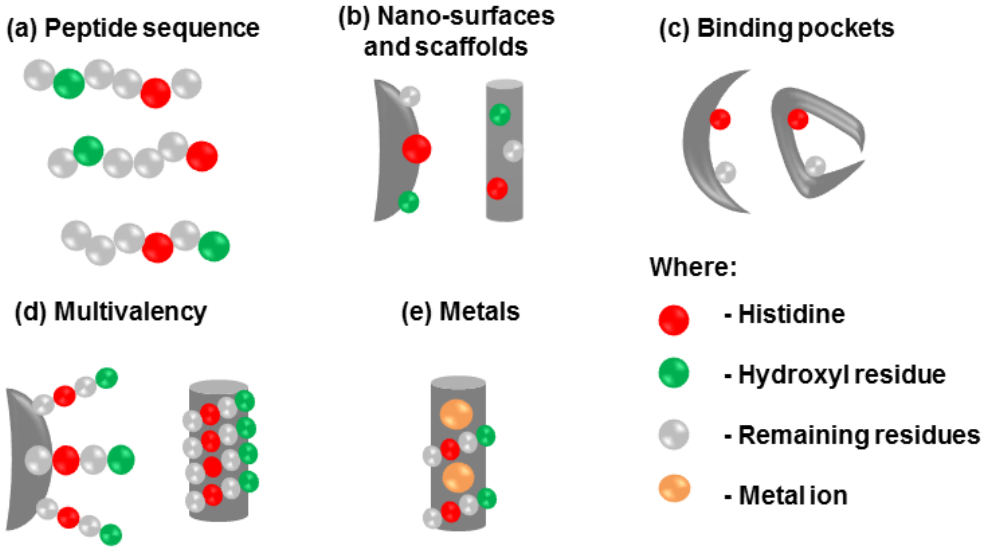


Figure 2. Approaches used in the mimicry of natural esterase enzymes.

A number of approaches have emerged in the pursuit of a synthetic catalyst (Figure 2). These approaches seek to mimic multiple aspects of biocatalysis, including (a) use of properties of primary peptide sequence to control the immediate chemical environment and functionality [8]. To further enhance activity, the peptides may be engineered into a designed environment, for example: (b) presentation of reactive sites for catalysis on scaffolds and particles [18,19] (c) design of binding pockets for substrates or transition states. Furthermore, (d) factors not normally associated with natural enzymes, such as multivalency may be considered and finally, (e) introduction of metal co-factors to enhance activity.

The first approach (a) is focussed on the inclusion of catalytic residues found in the active site of the natural enzyme. This approach takes into consideration primary sequence *i.e.* the relative positioning of chemical residues. This may have direct or indirect effects. Literature examples of designed esterases invariably include histidine that has a direct role in catalysis. Other residues involved in catalytic triads may also be included. It is clear that the amino acids directly surrounding this residue can further influence the catalytic activity by modulating the microenvironmental conditions, such as local pH or polarity to facilitate interactions with substrate molecules or transition states. The second approach (b) is focussed on utilising nano-surfaces and scaffolds for the presentation of catalytic and binding groups on the surface of a structure. This can be achieved using surfaces produced by self-assembly of peptide derivatives, or by using non-peptidic scaffolds, for example (inorganic) nanoparticles. These approaches may improve the catalytic activity due to the ordered presentation of catalytic moieties and the reduced dynamics of the system. Binding pockets (c) can be incorporated into synthetic structures to bind the transition state of the substrate and aid in catalysis. Commonly, these are hydrophobic surfaces or patches achieved by molecular self-assembly of amphiphilic peptides. Approach (d) focuses on multivalency. Although not normally found (at least not to the same extent) in naturally occurring proteins, designed systems offer the possibility of introducing multivalency in very large numbers, *i.e.* presentation of multiple catalytic residues and binding sites by using *e.g.* dendritic architectures. The final approach (e) focuses on the introduction of metal co-factors. These features have all been taken into account in our assessment of recent examples of synthetic, peptide based catalysts.

This review endeavors to summarize some of the more recent examples of the use of short peptides in biocatalysis with an emphasis on hydrolase (and mainly esterase) activity, as this is the most abundantly studied reaction where the natural enzyme it wants to mimic is also well understood. Specifically, we will investigate which of the above approaches gives rise to efficient catalytic activity and whether combinations can give rise to cooperative effects. If successful, synthetic mimics may provide an insight into the fundamental understanding of enzyme catalysis and pave the way towards rational design. This has the potential to result in robust catalysts for industrial applications but also designer catalysts for biotechnologies and potential therapeutic applications

that can interfere with biological pathways and facilitate the prevention and treatment of disease.

Primary Sequence in Catalysis

Chen and co-workers were first to explore the simplest mimic of a catalytic triad enzyme – by considering minimal sequences of the His, Asp, and Ser residues. What they discovered is surprising, in that the dipeptide Ser-His has the capability to cleave amide and ester bonds. The group reported that on incubation with Ser-His, cleavage was observed with linear and circular DNAs, proteins (BSA) and carboxyl ester substrate *p*-nitrophenyl acetate (*p*-NPA) [8]. These processes occur over a wide range of physical and chemical conditions. These authors also studied related oligopeptides, flanking the Ser-His motif with glycine and aspartic acid in different sequences or replacing serine or histidine (Figure 3).

It has been demonstrated that the single amino acid components of the triad, including histidine, in isolation do not exhibit cleavage activity, suggesting that they have to be covalently linked for cleavage to occur. The reverse sequence, His-Ser, is also inactive, suggesting a possible role for the terminal amine and acid groups in proximity to the imidazole and nucleophilic OH. Mutating these short sequences by replacing either histidine or serine with an alternative amino acid, results in the loss of cleavage activity. There are two exceptions; when serine is replaced with cysteine or threonine, however the activity is still lower than the Ser-His dyad. The former has a sulfhydryl side chain that has the potential to mirror the nucleophilic nature of the hydroxyl group on serine. Histidine was found to be essential for catalysis as all activity is lost upon the mutation of this residue. Residues can be added to the C terminus of the peptide or *in situ* while maintaining 40-60% of the original activity. Finally, with aspartic acid (Asp) attached to form the Ser-His-Asp tripeptide, the catalytic activity is at least as efficient as that of the dyad. The work demonstrates the peptide's requirement of a terminal acid or a neighbouring acidic side chain to assist in the catalysis mechanism. Strong evidence has been presented that it is the dipeptide itself that is responsible for the cleavage activities, implicating that the serine hydroxyl side chain and the histidine imidazole side chain have key mechanistic roles.

In addition to protein and DNA cleavage activity, it has also been reported that short peptides have the ability to catalyse peptide bond formation through trans-acylation. Luisi and co-workers described how Ser-His can catalyse the synthesis of a peptide bond from activated amino acid precursors, with comparable yields to chymotrypsin however on a time-scale which was approximately 6 times longer compared to the enzyme [20]. Upon incubation with the single amino acids and the isomer His-Ser, no peptide product was observed over the same time-frame and under the same conditions. The reaction is pH dependant with hydrolytic activity increasing in basic conditions, > pH 7. This study was considered to be of relevance to the chemical origin-of-life in that it seems reasonable that today's enzymes were preceded by simpler peptidic precursors.

Overall, these studies clearly demonstrate that even minimal primary sequences of relevant residues alone can influence the catalytic activity of that peptide. This includes the residues that make up the sequence but also the order in which they appear as demonstrated in the above examples. A core sequence, Ser-His, can be modified by adding residues to the termini of the

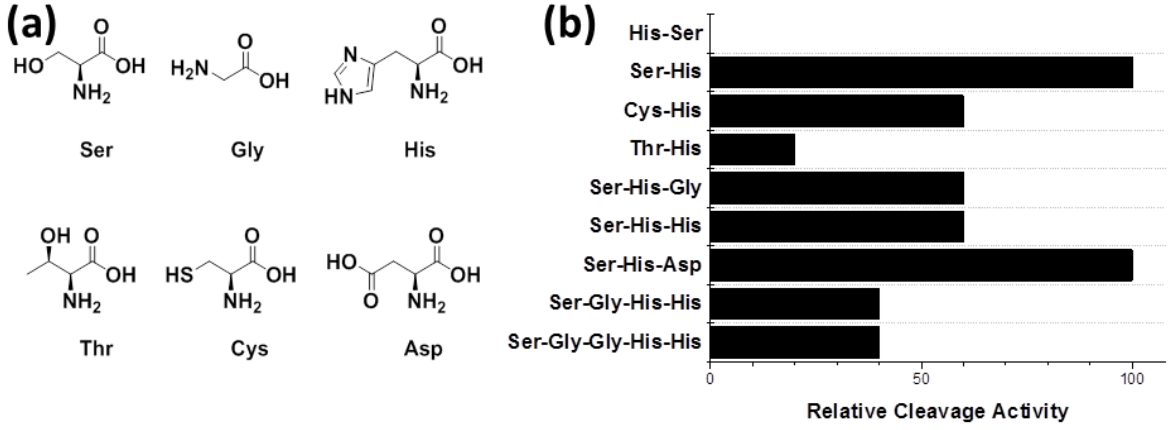


Figure 3. (a) Chemical structures of the peptides studied. (b) DNA and protein cleavage of Ser-His and related oligopeptides [8].

sequence with minimal loss of catalytic activity however no additional amino acids had been found to enhance the observed activity. Unfortunately no rates were reported for these examples so it is currently not possible to directly compare their catalytic activity with those of other design approaches.

Self-Assembling Peptides in Catalysis

The ability of certain peptide-based molecules to self-assemble into ordered nanostructures is now well established [21-26]. These often highly ordered structures can be chemically modified through choice of amino acids and may therefore be ideally suited for the design of catalysts. Due to the nature of the interactions within these structures; hydrogen bonding, hydrophobic interactions, π-π stacking *etc.*, the resulting materials may possess features that are similar to those of natural enzymes (*e.g.* amphiphilicity of architectures as well as substrate recognition and specific microenvironments for catalysis to take place,) however they would be simpler and tuneable. In addition, they may add additional features that are not normally found in naturally occurring catalysts, including multivalency and multiple, cooperative active sites situated along the length of the self-assembling fibers. Therefore, supramolecular self-assemblies based on peptides have become of great interest to biochemists and bionanotechnologists to produce new biologically inspired catalysts.

Stupp and co-workers reported a nanostructured, peptide-based catalyst obtained by molecular self-assembly. They demonstrated ester hydrolysis catalysed by histidine residues positioned on the surface of these cylindrical nanostructures [27] (Figure 4a). The core peptide segment of the structures is Lys-Leu-Leu-Leu-Ala-Ala-Ala with histidine incorporated on the lysine amine side chain and N-terminus whilst an aliphatic tail is present to encourage formation of cylindrical structures. Four variations of this structure were synthesised in order to study esterase activity alongside control peptides (1 – 4) (Figure 4b).

Peptide 1 was the amphiphile used to create self-assembled nanofibers. Peptide 2 lacks the amphiphilic

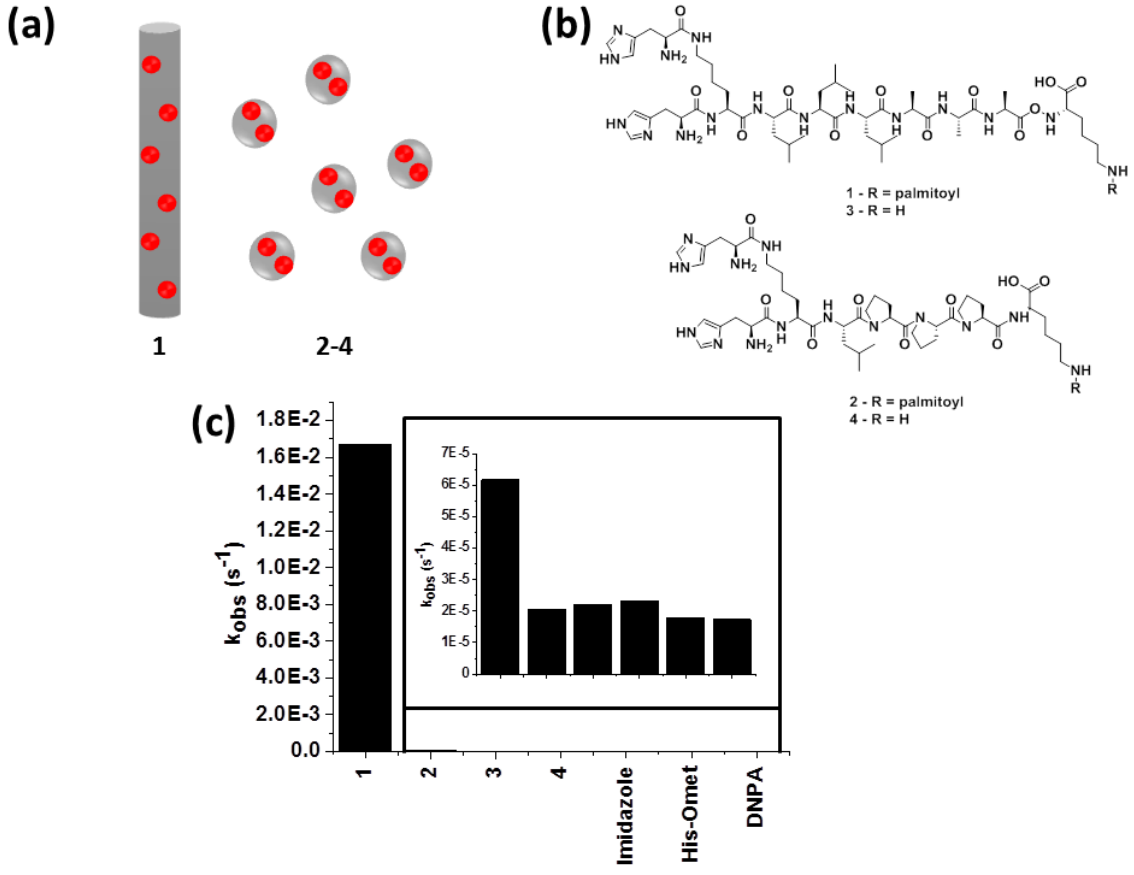


Figure 4. (a) Schematic of self-assembled peptide nanofiber with histidine residues present (peptide 1) and a spherical aggregate with histidine residues present (peptides 2-4) (b) Chemical structures of peptide amphiphiles used to build nanostructures. (c) kobs data of all peptides and controls.

tail to favour the formation of spherical aggregates whilst peptides 3 and 4 contain proline mutations preventing the formation of β-sheet structures and again resulting in spherical aggregate formation. On incubation with the esterase substrate (2,4-dinitrophenyl acetate), peptide 1 was found to have a k_{cat} of 1.67 + 0.13 10^{-2} s^{-1}, three orders of magnitude greater than for peptides 2-4 (Figure 4c). The nanostructure formed by peptide amphiphile 1 is a highly ordered architecture which presents numerous reactive sites on the surface of this structure, utilising multivalency, and it is thought that this highly-ordered structure is key for efficient catalysis.

As an alternative to aliphatic peptide amphiphiles, aromatic peptides amphiphiles are made up of a short peptide sequence capped by an aromatic moiety [25]. For these molecules, self-assembly is based upon aromatic stacking interactions between the aromatic moieties and the β-sheet-like hydrogen bonding arrangement between the peptides [28]. Liu and co-workers reported functionalised nanotubes based on aromatic peptide amphiphiles as a hydrolase model [29].

Fmoc-Phe-Phe-His, based on the well-known Fmoc-Phe-Phe molecule [25,30], self-assembles to form uniform nanotubes with the capability to hydrolyse the esterase substrate *p*-nitrophenyl acetate (*p*NPA) through catalytic centres present as a part of the molecule (Figure 5a). These structures also feature highly ordered β-sheet morphologies, determined by circular dichroism (CD) and fourier transform infrared spectroscopy (FTIR). To investigate the role of chemical environment/primary structure in the mechanism of catalytic nanostructures, Fmoc-Phe-Phe-Arg (Figure 5b) was also incorporated into the nanotubes in an effort to provide stable transition state binding sites in the form of guanidyl groups through the method of co-assembly. A co-assembled nanotube structure obtained *via* this method gave rise to a β-sheet structure which was uninterrupted by the incorporation of Fmoc-Phe-Phe-Arg (confirmed by Circular Dichroism, CD) [29]. The function of these structures can be controlled through co-assembly of two peptides, so that a single catalytic system can integrate various combinations of catalytic and binding sites [25].

Initial investigation into the Fmoc-Phe-Phe-His nanotubes reported a distinct increase in hydrolysis of the esterase substrate, *p*NPA, with both the Arg and His present. Upon co-assembly of the two molecules the rate of hydrolysis increases confirming that the co-assembled system is a more efficient catalyst, suggesting a role

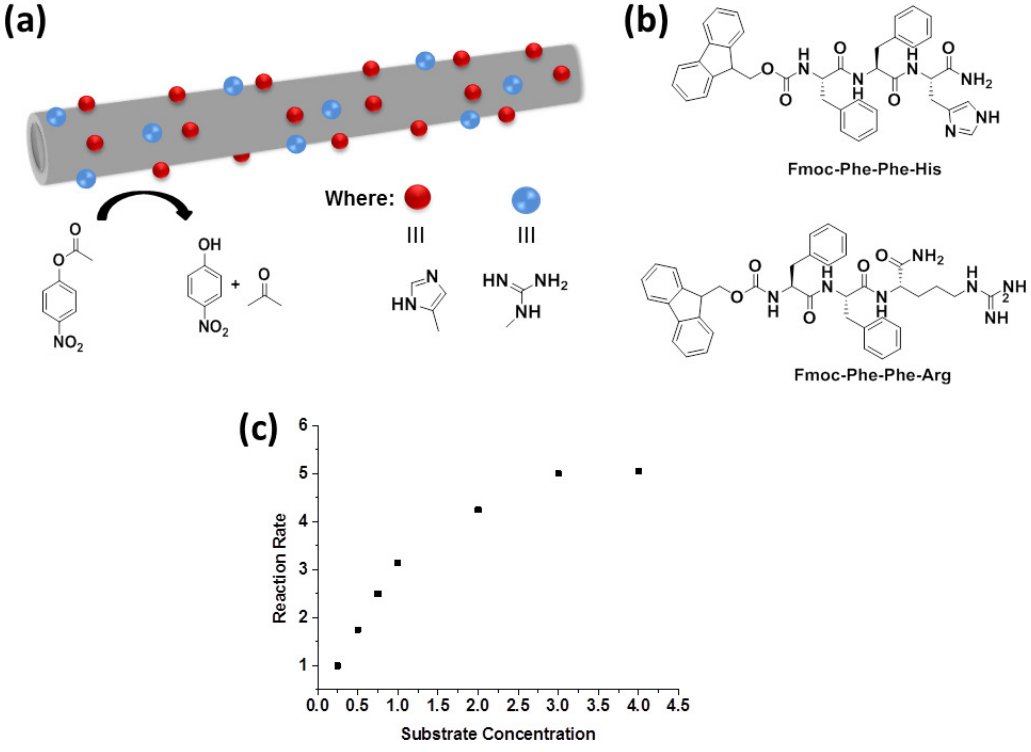


Figure 5. (a) Schematic of a co-assembled nanotube. Adapted from reference 29 with permission of The Royal Society of Chemistry. (b) Fmoc-Phe-Phe-His and Fmoc-Phe-Phe-Arg used to form co-assembly peptide nanotubes for a hydrolase model. (c) Schematic of Michaelis-menten plot of initial catalytic reaction rate vs. concentration of substrate as seen in reported data.

for transition state stabilisation (see Table 1). Typical enzyme kinetics resulted in a k_{cat} of 1.38 x 10^{-3} s^{-1} for the co-assembled system.

A further example that utilises peptide self-assembly to organise catalytic residues was published by Liang and co-workers [31]. Like the previous example, they make use of a self-assembling peptide to present catalytic centres (His) and binding sites (Arg) on the surface of a nanofiber. The self-assembling peptide (Q11) is salt-responsive and possesses the ability to self-assemble into a β-sheet nanostructure under mild conditions [32,33]. The self-assembling segment of the Q11 peptide is Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln [33,34], the N-terminus of the peptide is known to be presented on the surface of the fiber as shown by N-terminally biotinylated peptides, avidin-conjugated colloidal gold, and TEM – *i.e.* they should be available for catalysis [34]. Using co-assembly, functional residues can be arranged to gain the desired functionality (Figure 5, histidine and arginine side chains are present on the surface of a nanostructure) [34]. Peptides were synthesised with a terminal histidine residue (peptide Q11His) and with a terminal arginine residue as an electrostatic binding site (peptide Q11Arg). These peptides were then co-assembled to facilitate catalysis and substrate binding in the same sample.

Upon incubation of *p*-NPA with Q11His an enhancement in hydrolytic rate was observed. This increase in activity was proportional to catalyst concentration. This catalytic activity is believed to be due to the high density of catalytic residues presented on the surface of the fiber. The co-assembled system demonstrated a further enhancement in hydrolysis. Q11His had a value of 1.96 10^{-3} s^{-1} in comparison to 2.64 x 10^{-3} s^{-1} for Q11Arg/His (both of which are a marginal increase on the rate observed by Liu's co-assembled system at 1.38 x 10^{-3} s^{-1}). This suggests that the incorporation of an Arg residue is key to the increase in catalytic rate observed in the comparison of Q11His and Q11Arg/His.

As in the Fmoc-Phe-Phe-His/Arg system discussed above (Table 1 entry 3), the guanidyl group may act as an electrostatic binding site on the surface of the fiber which may be involved in stabilisation of the transition state. It is thought that the imidazole group of histidine activates a water molecule to produce a hydroxide ion which in turn attacks the carbonyl group of the substrate to generate a transition state, *i.e.* water acts as the nucleophile and assumes the role that serine OH plays in the enzyme. The transition state is stabilised by the guanidyl group, binding the oxide ions and activating the ester bond for cleavage before finally releasing the product. This

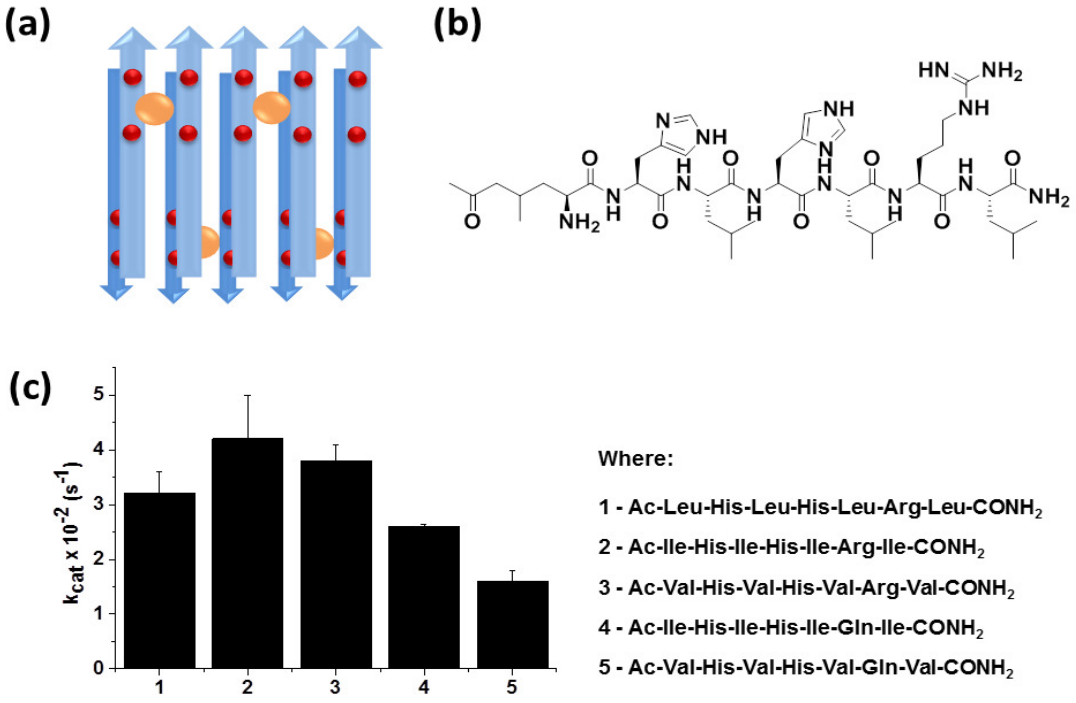


Figure 6. (a) Schematic of amyloid fibrils containing histidine residues and co-ordinating metal ions. (b) Structure of Ac-Leu-His-Leu-His-Leu-Arg-Leu-CONH2. (c) Esterase activity for designed peptides in the presence of 1 mM Zn2+.

proposed mechanism is based on kinetics parameters calculated from the Michaelis-Menten equation. The binding constant for Q11Arg/His (K_m) demonstrated a stronger binding affinity for the substrate for Q11Arg/His in comparison to Q11His. The incorporation of arginine results in an enhanced catalytic rate for Q11Arg/His.

Korendovych *et al.* reported that heptapeptides formed from amyloid structures have efficient ester hydrolysis activity in the presence of metal cofactors [35]. The sequence Leu-Lys-Leu-Lys-Leu-Lys-Leu was used as a template before design modifications were introduced to develop rational mutants due to the alternating hydrophobic residues within the sequence and the ability of these to interact further by hydrophobic interactions with other β-sheets (Figure 6a). Sequence mutation took place, retaining the apolar residues to drive the self-assembly process whilst the side chains of lysine were mutated to different polar functionalities. This gave the molecule the potential to support transition metal binding and catalysis.

Ac-Leu-His-Leu-His-Leu-Arg-Leu-$CONH_2$ (Figure 6b) has an esterase activity of k_{cat} 3.2 + 0.4 x 10^{-2} s^{-1} (Figure 6c) which was dependent on Zn^{2+} ions being present in the role of a cofactor (Figure 6). Histidine residues within the sequence support a tetrahedral zinc coordination sphere. This catalytic activity is also dependent on the nature of the residue at position 6, in this case arginine. The arginine residue plays a significant role in catalysis, related to the earlier described examples. Remarkably, upon replacement of the arginine residue with glutamine, the catalytic activity was reported to increase with regard to k_{cat}/K_M. This was the most active combination of residues. On mutation with aspartic acid, glutamic acid or histidine at position 6, the peptide had little or no activity.

The mutation of arginine with glutamine was intriguing due to an increase in observed catalytic activity. This new sequence was further mutated replacing the leucine residue with isoleucine or valine which is more susceptible to forming β-sheet structures. This resulted in the catalytic activity doubling in comparison to the original sequence Ac-Leu-His-Leu-His-Leu-Arg-Leu-$CONH_2$. On replacement of the leucine with alanine, a residue less likely to form the β-sheet structure, the catalytic activity decreased dramatically. The terminal acetyl and carboxamide groups were also reported to be important for catalysis to occur as upon the removal of these groups, the peptide is inactive for esterase activity. These changes in activity are not always expected and the combination of rational design and mutagenesis is a good way to improve catalytic activity. Co-ordination of the zinc ions is also crucial for catalysis to occur and also increases the catalytic activity compared to non-metal based esterase peptides that Hecht previously identified using combinatorial libraries[36,37] and as designed by Baltzer [38] and Mayo [39].

Finally, a recent article from Escuder *et al.* highlights a low molecular weight bola-amphiphile hydrogelator composed of an alkyl functionalised at both ends with a valine residue which is covalently linked to an imidazole moiety [40]. This gelator has proven to be an efficient catalyst once self-assembled and can hydrolyse *p*-NPA with k_{cat} calculated to range from 2.1 – 2.6 x 10^2 s^{-1} at pH 6-8. In this case, binding and catalysis takes place in reaction sites which are formed through non-covalent interactions. Further results hint at a role for binding and proposed contribution of both protonated (normally inactive) and non-protonated imidazole in a cooperative fashion, given the limited effect of pH on catalytic activity. This cooperativity may be a result of the unique self-assembly architecture used here and it is remarkable that catalytic activity (k_{cat}) is enhanced by several orders of magnitude compared to other examples (see Table 1).

In summary, (Table 1), the co-assembled Fmoc-Phe-Phe-His/Fmoc-Phe-Phe-Arg structure presents the lowest activity in comparison to the peptide amphiphile and the amyloid structures. It is possible that there are less catalytic reactive sites exposed on the surface of this nanostructure in comparison to the other examples and as a result the catalytic activity is slightly reduced. The importance of internal order of a structure is highlighted by the three orders of magnitude difference between Stupp's peptide amphiphile and his spherical aggregate samples. We also gain an insight into the importance of the co-ordination of metal ions in Korendovych's study of amyloid structures. In the presence of Zn^{2+} we see a significant rate enhancement over background activity resulting in the most efficient example of an enzyme mimic reported in this section. However, in the absence of the zinc ions, activity is reported as barely higher than that of background hydrolysis. These examples highlight some of the mimicry approaches which slightly enhance catalytic activity; however, the examples reported here are all within an order of magnitude of each other. Remarkably, the most significant rate enhancement is observed in a comparative system composed of a bolaamphiphile functionalised with Val-imidazole residues at both termini. It is proposed that protonated and non-protonated imidazole operate in a cooperative fashion to enhance activity. Overall, we can conclude so far that a combination of design approaches can be beneficial in the design of synthetic enzyme mimics.

Table 1. kcat values for self-assembling peptide structures.[1]

Entry		Substrate	Peptide Catalyst	kcat (s-1)	Mimicry Approaches
1	Stupp *et al.* [27]	DNPA	Peptide amphiphile	$1.67 + 0.13 \times 10^{-2}$	a, b, d
2	Stupp *et al. [27]*	DNPA	Peptide amphiphile (non-assembling)	$2.21 - 6.17 \times 10^{-5}$	a
3	Liu *et al.* [29]	*p*NPA	Co-assembly system	1.38×10^{-3}	a, b, c, d
4	Liang *et al. [31]*	*p*NPA	Q11Arg/His	2.64×10^{-3}	a, b, c, d
5	Korendovych *et al.* [35]	*p*NPA	Amyloid structures (with Zn^{2+})	$1.6 - 4.2 + 0.8 \times 10^{-2}$	a, b, e
6	Korendovych *et al.* [35]	*p*NPA	Amyloid structures (without Zn^{2+})	-	a, b
7	Escuder *et al. [40]*	*p*NPA	Low Molecular Weight Gelator	$2.1 - 2.6\ 10^{2}$	a, c
8	Prins *et al. [41]*	CBZ-Phe-ONP	$H_{(n)}$WDDD/Au MPC complex	$5.2 - 9.74 \times 10^{-3}$	a, b, d
9	Ulijn *et al.* [42]	*p*NPA	Phage + peptide	2.8 – 4.9*	a, b, d

1 The reaction conditions vary between study therefore they are summarised here for comparison. Stupp - hydrolysis of DNPA (varying concentrations) by 1×10^{-5} M imidazole functionalised molecules at 25°C in pH 7.4 50 mM HEPES buffer monitored by UV-Vis at 400 nm. Liu - hydrolysis of *p*NPA (0.5 mM) by 0.1 mM peptide nanotube catalyst at 25°C in pH 7.4 10 mM HEPES buffer monitored by UV-Vis at 400 nm. Liang – hydrolysis of *p*NPA (varying μM) by 200 μM of Q11His or Q11Arg/His in 1X PBS monitored by UV-Vis at 400 nm. Korendovych - hydrolysis of *p*NPA (0.195 - 0.75 mM) by 24 μM peptide at 22°C in pH 8.0 25 mM Tris buffer monitored by UV-Vis at 405 nm. Escuder – hydrolysis of *p*NPA (0-0.006 M) by 17×10^{-5} M low molecular weight gelator in 0.1 M Tris-HCl monitored by UV-Vis at 400 nm. Prins – hydrolysis of *N*-Cbz-D-Phe-ONP (10 μM) by peptides $H_0 - H_3$ (11 μM for H_0 and H_1; 8.5 μM for H_2 and H_3) in the presence of Au MPC 1 (60 μM) and HEPES (10 mM, pH 7.0) in H_2O:CH_3CN = 9:1 at 37 °C, monitored UV-Vis at 400 nm for 50 minutes. Ulijn – hydrolysis of *p*NPA (6 mM) by 0.01667 μM phage at 25°C in pH 7.4 PBS monitored by UV-Vis at 400 nm for 10 minutes.*lack of saturation kinetics precludes identification of k_{cat}, so k_{obs} is presented instead.

Peptide Functionalised Nanoparticles in Catalysis

As discussed in the previous section, peptides have the ability to form nanoscale structures and can provide chemical functionality resulting in designed systems with desired properties. The combination of peptide assembly and inorganic nanoparticles enables another route to produce nanomaterials which may possess catalytic activity as they fulfill the requirements of order, presentation of chemical moieties plus multivalency [27,29,35,43,44]. Nanoparticles decorated with functional ligands are particularly interesting in the development of catalysts based on multivalency. As with structures described in the previous section, self-assembly may be relied on for the formation of multivalent structures and is a pre-requisite for catalysis for many peptides that are not active on their own in solution. These self-assembled monolayers are of a relatively low-complexity in comparison to enzymes. The surface not only brings the catalyst and the substrate into close proximity, it also generates a micro-environment with a reduced local pH which activates the catalysis.

Prins and co-workers reported on histidine containing peptides which self-assemble on the surface of gold nanoparticles to give gold mono-layer protected clusters which possess esterase activity [41]. They previously reported that histidine residues within parts of a self-assembled mono-layer on the surface of a gold nanoparticle can act as a catalyst for transesterification reactions in a methanol-water solution [45]. In a more recent paper, they bring catalytic units together through co-assembly (Figure 7).

The peptides were designed around having three aspartic residues as anchors to attach the peptide to the functionalised nanoparticle *via* electrostatic interactions, the presence of tryptophan as a fluorescent label to confirm peptide binding and the presence of catalytic histidine residues (Figure 7a). On incubation with the substrate benzyloxycarbonyl-phenylalanine-4-nitrophenyl ester (Cbz-Phe-ONP) all complexes, $H_1 - H_3$/gold mono-layer protected clusters, demonstrated an increase in activity of two orders of magnitude relative to the background control sample. k_{obs} values were calculated to be $5.2 - 9.74 \times 10^{-3}$ s^{-1} (Figure 7b). The peptides free from the complex state showed no activity; confirming that the gold mono-layered peptide complexes are necessary to trigger catalytic activity.

Kinetic profiles were obtained and, upon fitting of experimental data to the appropriate model, provided

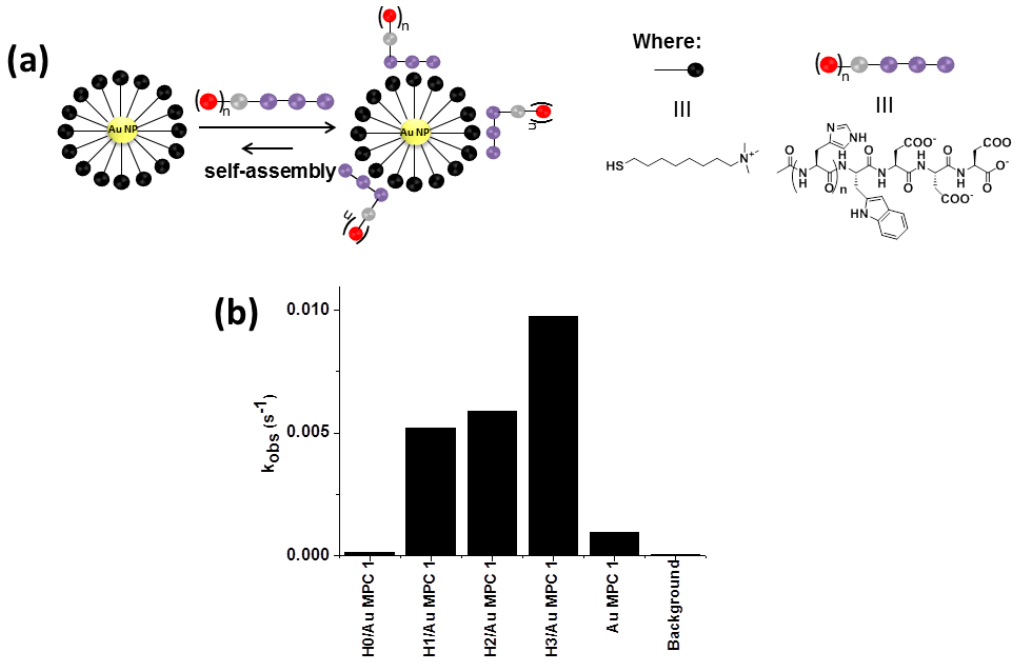


Figure 7. (a) Self-assembling peptides on the surface of gold monolayer-protected clusters which catalyse transesterification. Adapted with permission from reference J. Am. Chem. Soc., 2012, 134, 8396-8399. Copyright 2012 American Chemical Society**. (b) kobs rates for the gold mono-layer protected clusters.**

pseudo-first order rate constants with regards to catalytic activity. In order to make a direct comparison between peptides, the pseudo-first order rates were divided by for the peptide concentration to yield second order rate constants. A plot of the second order rate constants as a function of the number of histidines in the peptide sequence gave a straight line confirming that histidine residues are the origin of catalysis. It was reported that local pH of the monolayer/complex surface was higher than that of the bulk solution, indicating that catalysis through multivalent interactions across probes was unlikely to occur. Maximum catalytic activity per histidine residue was obtained when the peptide was on the surface of the cluster. As the peptide covers the surface of the nanoparticle, the carboxylates act as counter ions of the ammonium head groups decreasing the pH. The concentration of protonated imidazoles therefore increases, thereby facilitating catalysis.

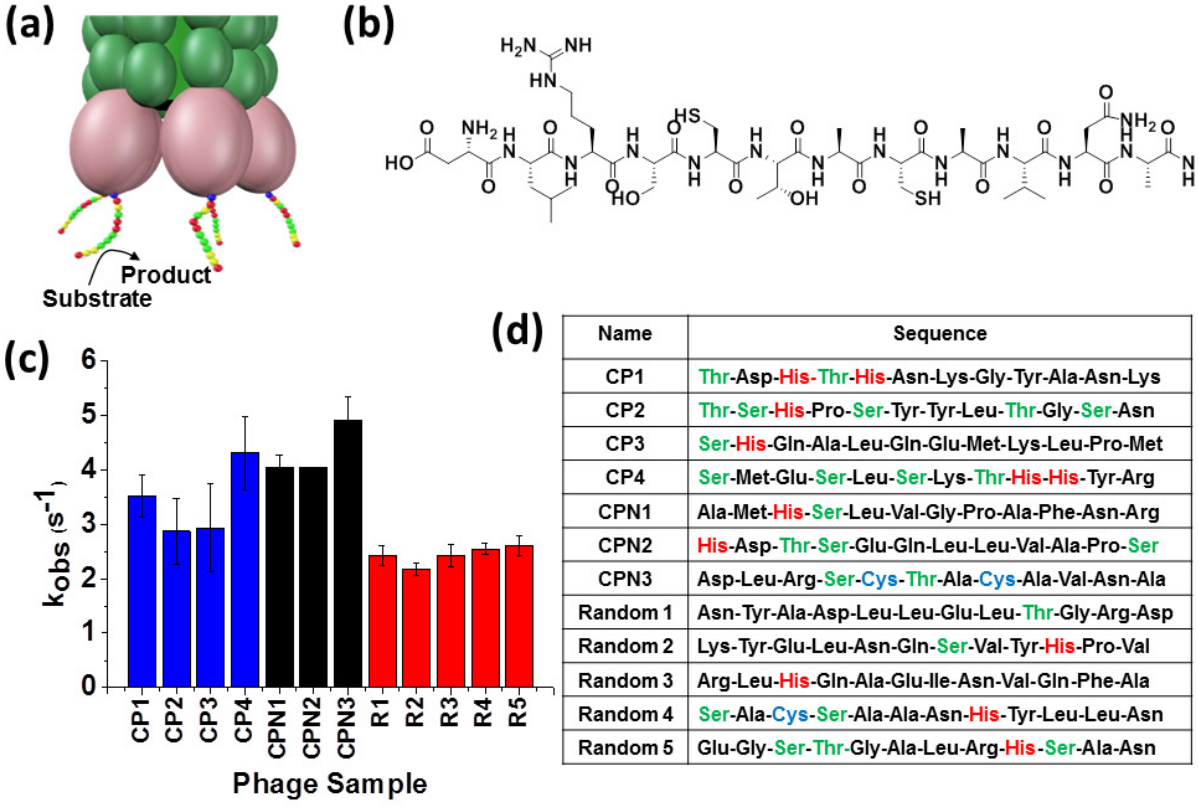
Further investigation into this system by Prins and co-workers yielded insights into the structural parameters that are necessary for catalysis to take place [46]. Once again, it has been demonstrated that the catalytic activity can be influenced by small structural changes in the primary peptide sequence of the supramolecular system. Mutation of the residues flanking histidine, from apolar to serine residues, results in an increase in catalysis rate. An investigation into substrate scope brought to light the finding that substrates that possess a hydrophobic component show increased levels of hydrolysis when incubated with the catalyst. This confirms that hydrophobic interactions with the hydrophobic section of the catalytic monolayer can play a key role in substrate binding. After four catalytic cycles while sequentially adding substrate, a decrease in catalytic rate is observed. It is hypothesised that this was due to competition between the carboxylate and the substrate.

The k_{cat} value for the $H_{(n)}$WDDD/Au MPC complex is in the same region as the self-assembled peptides discussed in the previous section albeit using a different substrate (the *p*-nitrophenol product is observed as before and the absorbance at 400 nm measured over time). Again, histidine residues were present in the peptide monolayer as catalytic residues and there was order in the assembled structure. The sequence of the peptide is significant to its activity. In contrast to the catalytic peptides mentioned previously, nanoparticles possess the advantage of mobility due to Brownian motion. Therefore, the active sites for catalysis can move with the nanoparticle and are not restricted as they are in self-assembled (gel) fibers.

Alternative Catalytic Systems

Whilst it is beneficial to design minimalistic biocatalysts that are suited to specific reactions and reaction conditions, the use of a discovery technique *e.g.* phage display, can identify potential catalysts from a vast library of potential sequences. Discovery techniques also directly relate sequence/structure to a particular, desirable function *i.e.* amide/ester hydrolysis. M13 phage display libraries are made up of viruses which display 5 copies of a single, short peptide (12 amino acids) on the tip of their protein coat. This technique can then be utilised to identify peptides of interest, the DNA can be sequenced to identify the peptide on display which in turn is synthesised for characterisation and mechanistic studies.

Recently, we have introduced a new screening method for the discovery of catalytic peptide sequences, making use of phage display [42]. The methodology enables unbiased discovery, through selection of filamentous phage particles which possess the ability to hydrolyse amide and ester bonds. This was based on a previously reported approach used to identify phages and peptides able to catalyse formation of inorganic particles [42]. Gel precursors, which upon catalytic action form self-assembling structures, were selected for the panning process. These precursors were then incubated with a phage library, phage virus particles with 5 copies of a single peptide displayed on the tip (Figure 8a), made up of 10^9 different peptide sequences. Any peptide sequences which possess the desired catalytic activity will produce the self-assembling molecule, resulting in formation of an aggregate at the tip and can therefore be extracted from the bulk phage library by centrifugation. With a vast number of combinations of peptide sequences, no assumptions regarding the origins of catalytic motifs can be made resulting in unbiased discovery and selection of catalytic viruses. The resulting phages can then be amplified by infection of an *E. Coli* host and have the potential to be mass produced for commercial applications. The selected phages were demonstrated to catalyse amide and ester hydrolysis. Catalytic rates reflect that, like in the Ser-His examples demonstrated by Chen et al., the lack of a well-defined binding pocket as compared to natural protease enzymes does not preclude catalytic activity.



Name	Sequence
CP1	Thr-Asp-His-Thr-His-Asn-Lys-Gly-Tyr-Ala-Asn-Lys
CP2	Thr-Ser-His-Pro-Ser-Tyr-Tyr-Leu-Thr-Gly-Ser-Asn
CP3	Ser-His-Gln-Ala-Leu-Gln-Glu-Met-Lys-Leu-Pro-Met
CP4	Ser-Met-Glu-Ser-Leu-Ser-Lys-Thr-His-His-Tyr-Arg
CPN1	Ala-Met-His-Ser-Leu-Val-Gly-Pro-Ala-Phe-Asn-Arg
CPN2	His-Asp-Thr-Ser-Glu-Gln-Leu-Leu-Val-Ala-Pro-Ser
CPN3	Asp-Leu-Arg-Ser-Cys-Thr-Ala-Cys-Ala-Val-Asn-Ala
Random 1	Asn-Tyr-Ala-Asp-Leu-Leu-Glu-Leu-Thr-Gly-Arg-Asp
Random 2	Lys-Tyr-Glu-Leu-Asn-Gln-Ser-Val-Tyr-His-Pro-Val
Random 3	Arg-Leu-His-Gln-Ala-Glu-Ile-Asn-Val-Gln-Phe-Ala
Random 4	Ser-Ala-Cys-Ser-Ala-Ala-Asn-His-Tyr-Leu-Leu-Asn
Random 5	Glu-Gly-Ser-Thr-Gly-Ala-Leu-Arg-His-Ser-Ala-Asn

Figure 8. Catalytic Phage. (a) Schematic of substrate conversion to product at the tip of the phage. (b) Structure of best esterase peptide on phage. (c) kobs values of the ester hydrolysis by various catalytic phages. (d) Peptide sequences on the tips of the phage. Green residues – hydroxyl groups, red residues – histidine and blue residues – cysteine.[42]

The esterase activity of these phages was investigated using *p*-nitrophenyl acetate. The CP peptides were obtained from panning using Fmoc-Thr and Leu-OMe. However, enzymes that possess amidase activity also possess the ability to hydrolyse esters. This raised concerns due to the potential of the methyl ester bond being broken causing degradation and diffusion of the reaction product away from the tip of the phage. As a result of this concern, the panning was repeated using Fmoc-Thr and Leu-NH_2 to eliminate this possibility of degradation and diffusion. This second panning experiment yielded CPN peptides. CPN3 (Figure 8b) showed the highest rate of hydrolysis, 4.9 s^{-1}, which was 2.5 times higher than the background rate. The rest of the phages followed in the order CP4 = CPN1,2 >CP1. The phages CP2 and CP3 had rates that were negligible in comparison to the background rate (Figure 8c). Control samples, R1-5, also demonstrate activity in the assay which is possibly related to the presence of histidine residues contained in the PIII protein domain of the phage and the well-known catalytic activity of the imidazole side chain.

It can be noted that the esterase activity is dependent on peptide sequence, most sequences include a hydroxyl group and a catalytic residue. What is remarkable is that, histidine is not necessarily present in catalytic structures, for example CPN3 contains two cysteine residues and an arginine moiety but is absent of histidine and shows appreciable activity. The paper does not describe the mechanism by which the catalysis occurs due to the observed sequence diversity and the major undertaking to thoroughly investigate all of the possible sequences. Analysis of rates as a function of substrate concentration shows a near-linear relationship implying that substrate binding is not playing a key role in the mechanism of catalysis. The introduction of a binding pocket into the system, mimicking natural enzymes, could be one method to look at improving the catalytic rates of these phages. This technology has the potential to evolve the methods by which simple catalytic mimics of living systems can be identified. This experiment was also carried out using free peptides of the above sequences however, the removal of the peptide from the tip of the phage resulted in a substantial decrease in esterase activity.

Another recent example that utilises phage in the mimicry of enzymes comes from Belcher and co-workers [47]. They developed a robust catalyst for targeted reactions in unique environments that are not standard assay conditions. M13 bacteriophages were selected as a template for their design as they possess multivalency, thermostability, scalability and can be genetically modified. Unlike the above example, they sought to display catalytic motifs from the enzyme carbonic anhydrase on the major protein coat, the PVIII protein, which is repeated 2,700 times to encase the phage DNA. This provides the virus with thousands of copies of the catalytic motif across the entire coat protein therefore providing multiple opportunities for the catalysis of hydrolysis reactions to occur. The binding pocket of this structure is open therefore it possesses a wide substrate scope. The catalytic efficiency of this protein structure was studied in aqueous conditions (2% DMSO, 98% PBS), organic solvents (98% DMSO and 2% PBS) and at elevated temperatures, conditions which the natural enzyme could not withstand.

The active site of carbonic anhydrase, an efficient zinc-based metalloenzyme, possesses two histidine residues in its active site which are separated by a phenylalanine in an α-helical structure. A third histidine residue is situated in a β-sheet close by and the three histidines together bind the $zinc^{2+}$ ion. A hydroxyl ion or substrate occupies the 4^{th} coordination site of the metal ion. The natural geometry of the enzyme was replicated throughout the protein coat of the bacteriophage. A pair of histidine residues was encoded at fixed positions and surrounded by a random combination of other amino acids in 8 amino acids inserts at the N terminus of the coat protein which is displayed on the surface the phage. Deeper into the protein, a third histidine residue was inserted so that the n-terminal histidine residues would be in close proximity to the third residue on an adjacent PVIII protein. Clones from the generated libraries were observed for sequence similarity to the natural enzyme and from a sub-population of 10 sequences, clone Asp-Asp-Ala-His-Val-His-Trp-Glu was selected for further study. Hydrolysis studies were carried out in the presence of zinc sulphate.

There was a significant increase in hydrolysis of *p*-NPA in the presence of the clone. This suggests a significant coordination between the catalytic residues however the activity is still lower than that of the natural enzyme. Kinetics studies were carried out to obtain a k_{cat} value of 0.002 s^{-1} for the clone in comparison to the natural enzyme, 1.2 s^{-1}, implying that the rate of turnover is a far more important factor in this case when compared to substrate binding. This is in good agreement with Ulijn *et al* who also concluded that substrate binding does not play a role in ester hydrolysis with their catalytic phage and peptide. Finally, the surrounding amino acids that make up the insert and their interactions have not been designed for their purpose and in natural examples, they are known to play a role in catalytic activity. Their consideration would likely increase the catalytic behaviour of the phage. These bacteriophage are also robust in non-aqueous solvents,

including DMSO, and exhibit a 30 fold increase in catalytic efficiency in this solvent. They can also catalyse a range of substrates including *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl propionate (*p*-NPP), *p*-nitrophenyl butyrate (*p*-NPB) and *p*-nitrophenyl palmitate (*p*-NPPa). The clone hydrolysed *p*-NPB at half of the efficiency of *p*-NPA and again the non-aqueous reactions had a higher efficiency than the aqueous systems. This capability to react with substrates of varying size makes the clone an interesting alternative to established biocatalysts. The catalytic efficiency was found to increase with higher temperatures, up to 65°C, the bacteriophage was twice as active at higher temperatures in comparison to room temperature. The M13 capsid is a robust structure which provides a catalytic site which has the ability to catalyse reactions at high temperatures. This could prove useful in industrial reactions.

Conclusions

Efficient and effective catalysis of natural enzymes has long been an inspiration to chemists working to overcome the expense, complexity and reaction media limitations of these proteins and develop new simple, synthetic catalysts that can be modified to suit desired substrates/reaction conditions. The examples above illustrate that some head way has been made in this field and rules are beginning to come to light for the design of these designed catalysts. We have seen that histidine is a crucial residue for catalysis in the majority of systems. It has also been demonstrated that design of the peptides can be used to alter catalytic activity, enhance stability, and provide insights into the mechanisms of reactions. The most effective approaches seem to be based on primary peptide sequence, nano-surfaces/scaffolds and multivalency with an especially impressive rate enhancement observed in a system where cooperative interactions between imidazoles were facilitated [40]. The presence of metal ions has also been shown to improve activity [35].

Self-assembly of nanostructures allows for the presentation of reactive sites on the surface of a highly ordered nanofiber scaffold. In comparison to spherical aggregate species the rate of hydrolysis of the catalytic nanostructures is significantly higher. The function of these structures can be taken advantage of, possibly through co-assembly, so that a single catalytic system can integrate various molecular recognition events.

Multivalent surfaces play a critical role in tuning the catalytic activity. New methodology for the design of efficient catalysis of ester hydrolysis displaying multiple functional residues on the surface of the fiber provides opportunities to design artificial enzyme mimics through the incorporation of residues with catalytic activity. The surface not only brings the catalyst and the substrate into close proximity, it also generates a micro-environment which may have a local pH which activates the peptide. The activity of the peptide can be influenced by the flanking residues of catalytic histidine residues. Therefore, the properties of a complex catalytic system can be controlled by small structural changes. Reduced efficiency of these systems after multiple turnovers could be due to the competition between carboxylates and the substrates. It can also be seen that metal ions have the potential to increase activity; in some cases by interacting with metal ion binding sites.

We have also seen that it is not only peptides that are being utilised as biocatalysts incorporating short peptides. M13 bacteriophages provide a new template to explore and work with in this field. Two examples discussed above describe bacteriophage with catalytic capability; however, the methods to achieve this end result are completely different, one *via* rational design and genetic modification, the other a screening process. Both systems demonstrated multivalency by displaying catalytic moieties in multiple positions and again histidine is present in the majority of samples; although, not all of them. These viruses are a great platform from which to spring into a new direction of enzyme mimicry and they have the potential to overcome severe drawbacks of natural enzymes including complexity and stability.

In conclusion, all reported methods that have been utilised in short peptide biocatalysis, both screening and design, result in very similar rates of hydrolysis which are still orders of magnitude lower than that of natural enzymes. However, it is clear that significant advancements are being made in the field of short peptides in biocatalysis. The designs featured here are promising and inspiring for future work into this area and for the development of future *de novo* peptide biocatalysts. In particular, supramolecular chemistry approaches offer opportunities to combine several design criteria, including those not found in natural enzymes. Identification of short peptide biocatalysts that rival enzymes may be a matter of time.

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